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Isolation and Characterization of Acetyl-Coenzyme A Synthetase from *Methanothrix soehngenii*

MIKE S. M. JETTEN, ALFONS J. M. STAMS,* AND ALEXANDER J. B. ZEHNDER

Department of Microbiology, Agricultural University Wageningen, NL 6703 CT Wageningen, The Netherlands

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In *Methanothrix soehngenii*, acetate is activated to acetyl-coenzyme A (acetyl-CoA) by an acetyl-CoA synthetase. Cell extracts contained high activities of adenylate kinase and pyrophosphatase, but no activities of a pyrophosphate:AMP and pyrophosphate:ADP phosphotransferase, indicating that the activation of 1 acetate in *Methanothrix* requires 2 ATP. Acetyl-CoA synthetase was purified 22-fold in four steps to apparent homogeneity. The native molecular mass of the enzyme from *M. soehngenii* estimated by gel filtration was 148 kilodaltons (kDa). The enzyme was composed of two subunits with a molecular mass of 73 kDa in an α_2 oligomeric structure. The acetyl-CoA synthetase constituted up to 4% of the soluble cell protein. At the optimum pH of 8.5, the V_{max} was 55 μmol of acetyl-CoA formed per min per mg of protein. Analysis of enzyme kinetic properties revealed a K_m of 0.86 mM for acetate and 48 μM for coenzyme A. With varying amounts of ATP, weak sigmoidal kinetic was observed. The Hill plot gave a slope of 1.58 ± 0.12, suggesting two interacting substrate sites for the ATP. The kinetic properties of the acetyl-CoA synthetase can explain the high affinity for acetate of *Methanothrix soehngenii*.

The terminal step in the breakdown of organic polymers under methanogenic conditions is the conversion of H_2/CO_2 or acetate by methanogens (9). The most abundant methanogenic substrate under these conditions is acetate (9, 33). Only two genera of methanogenic bacteria, *Methanosarcina* and *Methanothrix*, are capable of metabolizing acetate to methane. *Methanosarcina* spp., the most versatile methane bacteria, can use several compounds (H_2/CO_2, methanol, methylamines, and acetate) as growth substrates (11, 15, 18, 32). Its affinity for acetate is rather low (K_s, 5 mM), the growth yield is 2.1 g (dry weight) per mol of acetate, and the doubling time is 2 days (11, 32). *Methanothrix* can use acetate as a sole growth substrate. The growth yield (1.4 g/mol of acetate) and the growth rate (doubling time, 7 days) are low, but its affinity for acetate is high (K_s, 0.5 mM) (10, 40).

The pathway for acetate degradation involves transfer of the methyl group of acetate to coenzyme M, forming methyl-coenzyme M (methyl-CoM), which is then reductively demethylated to methane (6, 7, 19, 21, 22, 24, 37). The actual cleavage of the carbon-carbon bond in acetate is proposed to be catalyzed by carbon monoxide dehydrogenase (1, 5, 8, 28). High activities of carbon monoxide dehydrogenase were found in both *Methanosarcina* and *Methanothrix* spp. grown on acetate (8, 12, 16, 17, 35). Prior to the cleavage of the carbon-carbon bond, acetate is proposed to be activated to acetyl-coenzyme A (acetyl-CoA) (1, 6, 34). Different mechanisms of acetate activation were found in the two aceticlastic methanogens. For *Methanosarcina* spp., high activities of acetate kinase (EC 2.7.2.1) and phosphate acetyltransferase (EC 2.3.1.8) were reported (1, 6, 34, 36). In *Methanothrix* spp., only an acetyl-CoA synthetase (acetate: CoA ligase [AMP forming], EC 6.2.1.1) was present (16, 26). Recently, the purification and properties of the acetate kinase of *Methanosarcina thermophila* were described (1). This report summarizes the purification and characterization of the acetyl-CoA synthetase from *Methanothrix soehngenii* and a comparison is made between the acetate-activating systems of *Methanosarcina* and *Methanothrix* spp.

* Corresponding author.

MATERIALS AND METHODS

Organism and cultivation. *Methanothrix soehngenii* (DMS 2139) was the Opfikon strain isolated by Huser et al. (10). The organism was mass cultured on 80 mM sodium acetate in 25-liter carboys containing 20 liters of the medium described previously (12). Cultures were incubated without stirring at 35°C in the dark under an 80% N_2–20% CO_2 gas phase. Cells were harvested at the late log phase by continuous centrifugation (Carl Padberg Zentrifugenbau GmbH, Lahr/Schwarzwald, Federal Republic of Germany), washed in 50 mM Tris hydrochloride (Tris-HCl) (pH 8.0), and stored under N_2 at −20°C.

Chemicals. All chemicals were at least of analytical grade. Acetyl-CoA and P_1,P_5-di(adenosine-5')-pentaphosphate were purchased from Sigma Chemical Co. (Amsterdam). All other biochemicals were obtained from Boehringer Mannheim (Almere, The Netherlands). Sodium dodecyl sulfate (SDS) and acrylamide were from Bio-Rad Laboratories (Utrecht, The Netherlands). Gases were purchased from Hoekloos (Schiedam, The Netherlands). Platina catalyst was a gift of BASF (Arnhem, The Netherlands). Mono-Q HR 5/5, Q-Sepharose, Phenyl Superose HR 5/5, Superose 6 HR 10/30, and molecular mass standards for gel filtration and polyacrylamide gel electrophoresis (PAGE) were obtained from Pharmacia Fine Chemicals (Woerden, The Netherlands).

Analytical methods. Protein was determined with Coomassie brilliant blue G250 as described by Bradford (2). Bovine serum albumin was used as the standard. The purity of the enzyme after various chromatographic steps was determined by SDS-PAGE following the method of Laemmli (20). Molecular mass standards were α-lactalbumin, 14.4 kilodaltons (kDa); trypsin inhibitor, 20.1 kDa; carbonic anhydrase, 30 kDa; ovalbumin, 43 kDa; bovine serum albumin, 67 kDa; and phosphorylase B, 94 kDa. Gels were stained with Coomassie brilliant blue R250. Native enzyme molecular mass was determined on Superose 6 HR 10/30

equilibrated with 50 mM Tris-HCl (pH 8.0) with thyroglobulin (669,000 Da), ferritin (445,000 Da), catalase (232,000 Da), and aldolase (158,000 Da) as standards.

Enzyme purification. The purification of the acetyl-CoA synthetase was regularly performed within 1 day. Unless indicated otherwise, all procedures were carried out aerobically at room temperature (± 19°C). A frozen cell paste was thawed, diluted with 50 mM Tris-HCl (pH 8.0) in a 1:3 ratio, disrupted by passing through a French pressure cell at 135 MPa, and centrifuged for 30 min at 30,000 × *g*. The supernatant contained about 20 mg of protein per ml and is referred to as the crude extract. Membrane fractions were prepared by centrifugation of the crude extract at 110,000 × *g* for 75 min. The crude extract (25 ml) was applied to a column (3.3 by 10 cm) of Q-Sepharose (fast flow) equilibrated with 50 mM Tris-HCl (pH 8.0) (buffer A). After the column was washed with 40 ml of buffer A, the adsorbed protein was eluted in a 500-ml linear gradient of 1 M NaCl in buffer A. The flow rate was 4 ml/min. Fractions (10 ml) were collected and analyzed for acetyl-CoA synthetase activity. The acetyl-CoA synthetase eluted at 0.15 M NaCl. Fractions with activities higher than 2 U/mg were pooled and desalted in an Amicon ultrafiltration cell (Grace, Rotterdam, The Netherlands) with a PM 30 filter. The remaining steps in the purification were performed with a high-resolution fast protein liquid chromatography (FPLC) system (Pharmacia/LKB, Woerden, The Netherlands) equipped with a model 2152 LC controller. Repetitively, four samples of 5 ml of the concentrated desalted enzyme solution were injected onto a Mono-Q HR 5/5 anion-exchange column equilibrated with Tris-HCl, pH 9.0. A 12-ml linear gradient from 0 to 0.4 M of NaCl in Tris-HCl, pH 9.0, was applied at a flow rate of 1.0 ml/min. Fractions with acetyl-CoA synthetase activity were concentrated to 2.0 ml in a Centricon PM 30 (Grace, Rotterdam, The Netherlands). The enzyme solutions of four runs were combined and mixed in a 1:1 ratio with 2 M $(NH_4)_2SO_4$ in buffer A and applied to a Phenyl Superose HR 5/5 column. A 20-ml linear gradient from 1 to 0 M $(NH_4)_2SO_4$ in buffer A was applied at a flow rate of 0.5 ml/min. Fractions with acetyl-CoA synthase activity were concentrated to 2.0 ml in a Centricon PM 30. The concentrated enzyme solution was injected on a Superose 6 HR 10/30 gel filter equilibrated with buffer A. The column was developed at a flow rate of 0.5 ml/min. Purified acetyl-CoA synthetase was collected, concentrated in Centricon PM 30, frozen in liquid N_2, and stored at −80°C until use.

Assays. Acetyl-CoA synthetase (EC 6.2.1.1) was assayed either by following the formation of acetyl-CoA as hydroxamate from acetate, HSCoA, and ATP or by coupling the acetyl-CoA synthetase reaction with adenylate kinase, pyruvate kinase, and lactate dehydrogenase (25, 39). In the first assay, the standard reaction mixture included the following compounds (in micromoles per milliliter): ATP, 2; sodium acetate, 10; $MgCl_2$, 2; glutathione, 2; Tris-HCl (pH 8.5), 100; neutralized NH_2OH, 600; coenzyme A, 0.2; and enzyme. Acetyl-CoA was determined as the hydroxamate by the method of Rose et al. (27). For the second assay, the reaction mixture contained (in micromoles per milliliter): Tricine-KOH (pH 8.5), 100; $MgCl_2$ 4; phosphoenolpyruvate, 2; NADH, 0.4; ATP, 2; sodium acetate, 10; coenzyme A, 0.2; glutathione, 2.0; plus adenylate kinase, 1 U; pyruvate kinase, 0.8 U; lactate dehydrogenase, 3 U; and enzyme. The rate of NADH oxidation was followed continuously at 340 nm in an LKB/Biochrom Ultrospec K spectrophotometer. All incubations were done at 35°C. One unit of enzyme is defined as the amount which catalyzes the formation of 1 μmol of acetyl-CoA per min.

Acetate kinase (EC 2.7.2.1) and phosphate acetyltransferase (EC 2.3.1.8) concentrations were determined as described by Aceti and Ferry (1).

Adenylate kinase (EC 2.7.4.3) was measured photometrically by following the formation of ADP from AMP and ATP at 340 nm by coupling the reaction to the oxidation of NADH via pyruvate kinase and lactate dehydrogenase or by following the formation of ATP from ADP at 340 nm by coupling the reaction to the reduction of $NADP^+$ via hexokinase and glucose-6-phosphate dehydrogenase (25). The reaction mixture for the formation of ADP contained (in micromoles per milliliter): Tricine-KOH (pH 8.2), 100; $MgCl_2$ 4; phosphoenolpyruvate, 2; NADH, 0.4; ATP, 2; AMP, 2; glutathione, 2.0; plus pyruvate kinase, 0.8 U; and lactate dehydrogenase, 3 U. The reaction mixture for the formation of ATP contained (in micromoles per milliliter): Tricine-KOH (pH 8.2), 100; $MgCl_2$, 4; ADP, 4; glutathione, 2.0; $NADP^+$, 0.4; D-glucose, 100; plus glucose-6-phosphate dehydrogenase, 0.7 U; and hexokinase, 0.7 U.

Inorganic pyrophosphatase (EC 3.6.1.1) was measured by following the formation of P_i (14). The reaction mixture contained (in micromoles per milliliter): Tricine-KOH (pH 8.2), 100; $MgCl_2$, 4; sodium pyrophosphate, 5; and glutathione, 2. Samples (200 μl) were taken at 1-min time intervals and added to 100 μl of 5 M H_2SO_4 to stop the reaction. The precipitated protein was removed by centrifugation, and phosphate content was determined in the supernatant by the modified method of Fiske-SubbaRow as described by Josse (14).

Pyrophosphate:AMP phosphotransferase was measured by following the formation of ADP from AMP and PP_i by coupling the reaction to the reduction of $NADP^+$ via adenylate kinase, hexokinase, and glucose-6-phosphate dehydrogenase (38). The reaction mixture contained (in micromoles per milliliter): Tricine-KOH (pH 8.2), 100; $MgCl_2$, 4; PP_i, 20; $NADP^+$, 0.4; AMP, 4; D-glucose, 100; glutathione, 2.0; plus adenylate kinase, 1 U; hexokinase, 0.7 U; and glucose-6-phosphate dehydrogenase, 0.7 U.

Pyrophosphate:ADP phosphotransferase was measured by following the formation of ATP from ADP and PP_i by coupling the reaction to the reduction of $NADP^+$ via hexokinase and glucose-6-phosphate dehydrogenase. Adenylate kinase was inhibited by Ap_5A [P_1,P_5-di(adenosine-5′)-pentaphosphate). The reaction mixture contained (in micromoles per milliliter): Tricine-KOH (pH 8.2), 100; $MgCl_2$, 4; PP_i, 20; $NADP^+$, 0.4; AMP, 4; D-glucose, 100; glutathione, 2.0; Ap_5A, 0.2; plus hexokinase, 0.7 U; and glucose-6-phosphate dehydrogenase, 0.7 U (25).

ATPase (EC 3.6.1.3) content was determined by following formation of P_i from ATP. The reaction mixture contained (in micromoles per milliliter): Tricine-KOH (pH 8.2), 100; $MgCl_2$, 4; and sodium ATP, 5. Samples (200 μl) were taken at 1-min time intervals. The reaction was stopped by the addition of 100 μl of 5 M H_2SO_4. The precipitated protein was removed by centrifugation, and phosphate content was determined as described by Skrabanja et al. (31).

In all assays, an appropriate amount of cell extract was used. The reactions were started by the addition of cell extract or substrate.

The assays were performed under both strict anaerobic and aerobic conditions. Since no difference in activity was found under either condition, assays were routinely performed aerobically.

Kinetic analysis. The kinetic parameters of acetyl-CoA

TABLE 1. Enzyme activities in cell extract of *Methanothrix soehngenii*[a]

Enzyme	EC no.	Sp act (μmol/min per mg)
Acetyl-CoA synthetase	6.2.1.1	2.7 ± 0.6
Adenylate kinase	2.7.4.3	4.2 ± 0.7
Pyrophosphatase	3.6.1.1	0.92 ± 0.1
ATPase	3.6.1.3	0.041 ± 0.005
Acetate kinase	2.7.2.1	ND[b]
Phosphate acyltransferase	2.3.1.8	ND
PP_i:AMP phosphotransferase		ND
PP_i:ADP phosphotransferase		ND

[a] The values represent mean activities of at least four extract preparations. Enzymatic activity for each extract was the average of five determinations.
[b] ND, Not detected.

synthetase were determined at 35°C and pH 8.5. The continuous assay was used for the determination of the K_m and V_{max} for acetate and coenzyme A. The K_m for ATP was determined with the discontinuous assay. The inhibitory effects of AMP and PP_i on the acetyl-CoA synthetase were tested with the discontinuous assay at different AMP, PP_i, and ATP concentrations.

RESULTS

Acetate activation. Activities of enzymes possibly involved in the activation of acetate in *Methanothrix soehngenii* are summarized in Table 1. None of the enzyme activities was influenced by oxygen. A high activity of an acetyl-CoA synthetase and no acetate kinase activity were detected in cell extracts. Enzymes necessary for the conversion of AMP and PP_i, which are formed in the acetyl-CoA synthetase reaction, were also present in high levels. This confirms earlier findings made by Kohler and Zehnder (16). After ultracentrifugation, these enzymes were found in the soluble fraction, whereas the control enzyme ATPase was completely recovered in the particulate fraction. Since the energy in the PP_i represents metabolically useful energy, cell extracts were tested for the most obvious PP_i-dependent enzymes. No PP_i:AMP or PP_i:ADP phosphotransferase activities were found.

Enzyme purification. The acetate-activating enzymes in *Methanosarcina* and *Methanothrix* spp. were reported to be insensitive to molecular oxygen (1, 15, 16). The purification of the acetyl-CoA synthetase therefore required no strict anaerobic conditions. The purification was carried out at room temperature and generally took only 1 day. In four steps, a 22-fold-purified enzyme was obtained (Table 2). Q-Sepharose chromatography proved to be an effective first step in the purification of the acetyl-CoA synthetase, since the enzyme was one of the first proteins to elute from the column after application of the NaCl gradient. After concentration and desalting, the enzyme preparation was injected onto an FPLC system and purified to homogeneity. The loss in total activity as given in Table 2 mainly occurred because only those fractions with high specific activities were pooled in each purification step.

TABLE 2. Purification of acetyl-CoA synthetase of *Methanothrix soehngenii*

Step	Protein (mg)	Activity (U)	Sp act[a] (U/mg)	Purification (fold)	Yield (%)
Crude extract	400	880	2.2	1	100
Q-Sepharose	40	392	9.8	5	45
Mono-Q	16	272	17.2	8	30
Phenyl-Superose	8	192	24.1	11	22
Superose	1	50	48.3	22	6

[a] Micromoles of acetyl-CoA formed per minute per milligram of protein.

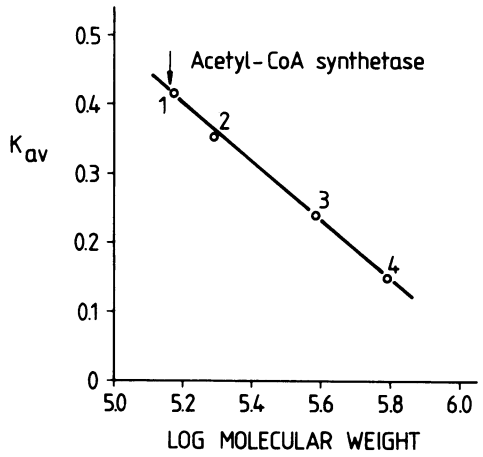

FIG. 1. Molecular mass estimation of the native acetyl-CoA synthetase on Superose 6 HR 10/30. Standards were aldolase (158 kDa, 1); catalase (232 kDa, 2); ferritin (446 kDa, 3); and thyroglobulin (669 kDa, 4). The position of acetyl-CoA synthetase is indicated by the arrow.

Characterization of the purified enzyme. The molecular mass of the native acetyl-CoA synthetase was estimated by gel filtration on Superose 6 HR 10/30 and appeared to be 148 kDa, compared with standards of known molecular mass (Fig. 1). SDS-PAGE of the purified enzyme revealed one subunit with relative molecular mass equal to 73 kDa, which suggests an α_2 subunit stoichiometry for the native enzyme (Fig. 2). The activity of the purified enzyme did not decrease significantly in the presence of air. The enzyme could be stored at −20 or −70°C without any loss of activity for at least 2 months. When the enzyme was kept at 4°C, aerobically or anaerobically, 50% of the activity was lost within 72 h.

Substrate specificity. In addition to acetate, a coenzyme A-dependent activation of some other organic acids was catalyzed to some extent by the purified enzyme (Table 3).

Kinetic properties. The reaction rate at different acetate and coenzyme A concentrations followed Michaelis-Menten kinetics. Half-maximal rates were obtained at 0.86 mM acetate and at 48 μM coenzyme A. The V_{max}, at the optimal pH of 8.5 (100 mM Tricine-KOH) and at 35°C, was 55 μmol of acetyl-CoA formed per min per mg of protein. With ATP, however, a weak sigmoidal velocity curve was found. The concentration of ATP which gave half-maximal rates was obtained from the double-reciprocal plot and appeared to be 1 mM. These data suggest cooperative binding of ATP, as reported for the acetate kinase of *Clostridium thermoaceticum* (29). A Hill plot of the data resulted in a Hill coefficient of 1.58 ± 0.12, suggesting two interacting substrate sites (Fig. 3) (29).

Inhibition studies. Acetyl-CoA synthetase was inhibited by the end products AMP and PP_i. When the activities at various concentrations of AMP, PP_i, and ATP were plotted by the method of Dixon (4), a K_i of 4 and 6.5 mM was determined for AMP and PP_i, respectively (Fig. 4A and B).

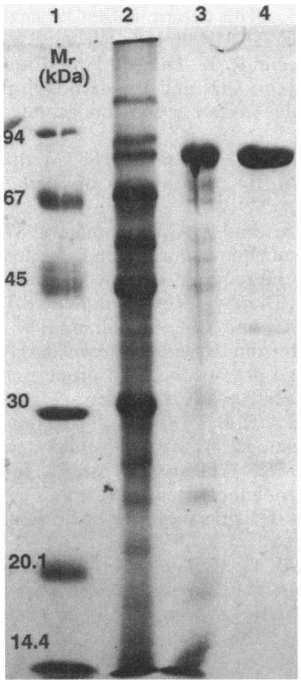

FIG. 2. SDS-PAGE of the different steps in the purification procedure. Lane 1, Molecular mass markers; lane 2, crude extract (80 μg); lane 3, pooled fractions from phenyl-Superose (30 μg); lane 4, pooled fractions from Superose 6 HR 10/30 (50 μg).

DISCUSSION

Methanosarcina and *Methanothrix* spp. have different enzyme systems for the activation of acetate. An acetate kinase and a phosphate acyltransferase convert acetate to acetyl-CoA in *Methanosarcina* spp., whereas this conversion is catalyzed by an acetyl-CoA synthetase in *Methanothrix* spp. (1, 16). The acetate kinase of *Methanosarcina thermophila* and the acetyl-CoA synthetase of *Methanothrix* spp. have now been purified, and their properties can be compared. Acetyl-CoA synthetase is an abundant protein of *Methanothrix soehngenii*. From the increase in specific activity upon purification and from the 6% recovery, it can be calculated that up to 4% of the soluble cell protein of *Methanothrix soehngenii* is acetyl-CoA synthetase. This level is somewhat higher than the 1% acetate kinase that can be calculated for *Methanosarcina thermophila* (1). The acetyl-CoA synthetase has a homodimeric subunit composition similar to that of the acetate kinase of *Methanosarcina*, but the size of the subunits is somewhat larger, 73 versus 58 kDa (1). Both enzymes exhibit the same temperature stability and are not sensitive to oxygen (1). Both enzymes are capable of activating some other fatty acids, like propionate (1). The rate of these conversions, however, is very low. The

TABLE 3. Substrate specificity of the purified acetyl-CoA synthetase

Substrate[a]	Relative activity[b] (%)
Acetate	100
Propionate	5
Butyrate	1.6
Benzoate	0.6
Valerate	0.3
Formate	0
Succinate	0

[a] 10 mM sodium salt was used in the assay.
[b] Relative to activity with acetate (100%; 54 μmol of acetyl-CoA formed per min per mg of protein).

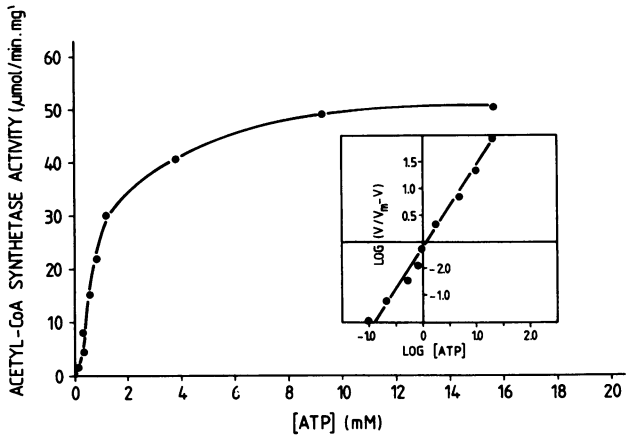

FIG. 3. Relationship between ATP concentration and activity of the purified acetyl-CoA synthetase. Conditions and calculations are described in Materials and Methods. Inset: Hill plot of the same data.

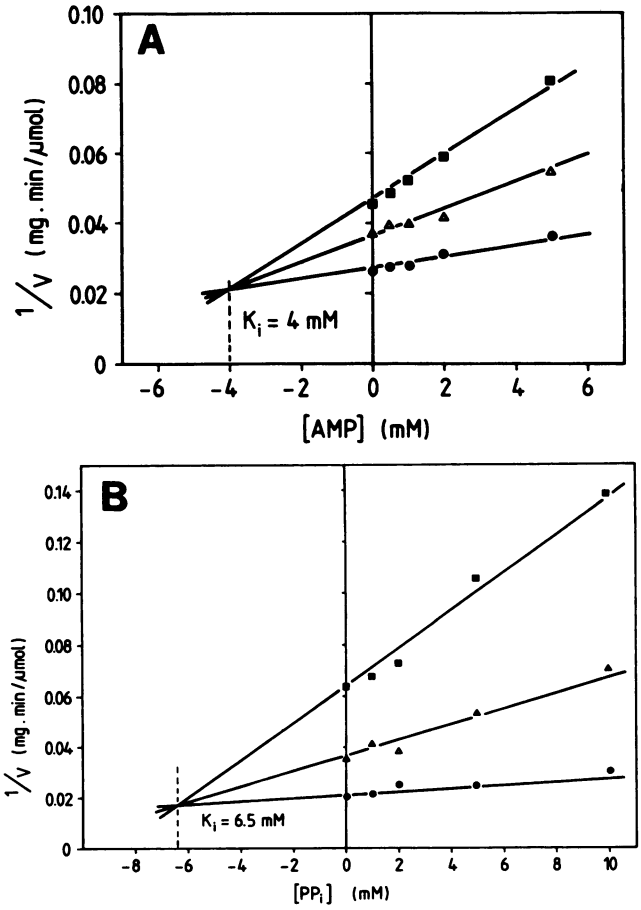

FIG. 4. Inhibition of acetyl-CoA synthetase by AMP (A) and PP_i (B). The data are plotted by the method of Dixon (4). ATP concentration: ●, 1 mM; △, 2 mM; ■, 5 mM.

major differences between the enzymes are the kinetic properties. The acetate kinase of *Methanosarcina thermophila* has a high V_{max} (660 U/mg), but a low affinity for acetate (K_m, 22 mM) (1). The acetyl-CoA synthetase of *Methanothrix soehngenii* has a high affinity for acetate (K_m, 0.8 mM), but has a lower V_{max} (55 U/mg). The acetate kinase exhibits normal Michaelis-Menten kinetics towards acetate and ATP. The acetyl-CoA synthetase, however, shows a weak sigmoidal velocity curve with varying amounts of ATP, which indicates the cooperative binding of ATP to the enzyme. The Hill plot gave a slope of 1.58, which suggests two interacting binding sites for ATP. This might enable the cell to regulate the acetate activation (30).

The differences in V_{max} and K_m value for acetate of the two acetate activating enzymes may explain the differences in the physiological properties and the ecological distribution of the two types of acetoclastic methanogens in nature. *Methanosarcina* spp., which have a high maximal specific growth rate and a low affinity for acetate, are dominant in environments with high acetate concentrations, whereas *Methanothrix* spp., which have the reverse properties, are most abundant in environments with low acetate concentrations (9, 33). It cannot, however, be excluded that the favorable surface-volume ratio of *Methanothrix* spp. and differences in acetate uptake systems are additional factors of importance in the affinity for acetate (23). The two sets of acetate-activating enzymes were also found in *Escherichia coli* and function at different acetate concentration. At high acetate concentrations, acetate is activated with an acetate kinase-phosphate acyltransferase system, whereas studies with mutants showed that at low acetate concentrations, an acetyl-CoA synthetase activity is displayed (3, 36).

The presence of the acetyl-CoA synthetase-adenylate kinase-pyrophosphatase system in *Methanothrix soehngenii* implies that 2 ATP molecules are required for the activation of 1 molecule of acetate. No enzymes were detected which make use of the energy present in the PP_i bond. It is possible that these enzymes were inactivated or that the hydrolysis of PP_i is just needed to pull the activation process at low acetate concentrations. It is rather intriguing how *Methanothrix* is able to generate metabolic energy for growth, especially because it was postulated that the acetoclastic cleavage only yields 1 ATP (6, 36, 37). It has to be ensured, therefore, that the transfer of electrons formed in the oxidation of the enzyme-bound carbonyl moiety to the methyl-CoM reductase forms an electrochemical gradient which is high enough to enable the synthesis of more than 2 ATP. Future research is concentrated on the elucidation of the electron transfer processes in *Methanothrix soehngenii*.

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